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# The cxc chemokine cCAF stimulates differentiation of fibroblasts into myofibroblasts and accelerates wound closure

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**C**hemokines are small cytokines primarily known for their roles in inflammation. More recently, however, they have been implicated in processes involved in development of the granulation tissue of wounds, but little is known about their functions during this process. Fibroblasts play key roles in this phase of healing: some fibroblasts differentiate into myofibroblasts,  $\alpha$ -smooth muscle actin (SMA)-producing cells that are important in wound closure and contraction. Here we show that the CXC chemokine chicken chemotactic and angiogenic factor (cCAF) stimulates fibroblasts to produce high levels of  $\alpha$ -SMA and to contract collagen gels more effectively than do normal fibroblasts, both characteristic properties of myofibroblasts. Specific inhibition of  $\alpha$ -SMA expression resulted in abrogation of cCAF-induced contraction. Furthermore, application of cCAF

to wounds in vivo increases the number of myofibroblasts present in the granulation tissue and accelerates wound closure and contraction. We also show that these effects in culture and in vivo can be achieved by a peptide containing the NH<sub>2</sub>-terminal 15 amino acids of the cCAF protein and that inhibition of  $\alpha$ -SMA expression also results in inhibition of N-peptide-induced collagen gel contraction. We propose that chemokines are major contributors for the differentiation of fibroblasts into myofibroblasts during formation of the repair tissue. Because myofibroblasts are important in many pathological conditions, and because chemokines and their receptors are amenable to pharmacological manipulations, chemokine stimulation of myofibroblast differentiation may have implications for modulation of functions of these cells in vivo.

## Introduction

Chemokines are primarily known for their roles in inflammatory and immune responses. However, these small cytokines have also been shown to be upregulated during development of the granulation tissue of wounds and to act on endothelial cells and keratinocytes, cells that perform important functions in the development of the healing tissue (Martins-Green and Bissell, 1990; Nanney et al., 1995; Luster et al., 1998; Martins-Green and Feugate, 1998). In addition to these cell types, fibroblasts are also critical players during formation of the granulation tissue. These cells are activated by cytokines and growth factors that are released from platelets and produced by macrophages. The activated fibroblasts proliferate and migrate across the provisional matrix formed by the fibrin-plasma fibronectin clot. As the clot is digested by plasmin, fibroblasts replace it with cellular fibronectin, tena-

scin, and collagen III (Mackie et al., 1988; Clark, 1993), molecules that are critical for migration of endothelial cells and keratinocytes into the wound and for proper scar formation. Some of the fibroblasts infiltrating the wound differentiate to become myofibroblasts (Gabbiani, 1996; Powell et al., 1999), cells that have bundles of  $\alpha$ -smooth muscle actin (SMA)\* and can contract, contributing to closure of the wound (Germain et al., 1994; Lanning et al., 2000).

Chemokines are small, positively charged, secreted proteins that consist of an NH<sub>2</sub>-terminal region of variable conformation followed by a loop, three antiparallel  $\beta$  strands, and a COOH-terminal  $\alpha$  helix (Clark-Lewis et al., 1995). They can be divided into four families based on the position of the first two cysteines. The two major families are the CXC family in which the two cysteines are separated by any single amino acid (e.g., cCAF, interleukin [IL]-8,

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Key words: chemokine; myofibroblast; wound healing; 9E3/cCAF;  $\alpha$ -smooth muscle actin

\*Abbreviations used in this paper: CAM, chicken chorioallantoic membrane; cCAF, chicken chemotactic and angiogenic factor; CEF, embryonic connective tissue fibroblast; IL, interleukin; MGSA, melanocyte growth-stimulating activity; ODN, oligodeoxynucleotide; SMA, smooth muscle actin.

gro $\alpha$ /melanocyte growth-stimulating activity [MGSA], SDF-1, PF4, IP-10) and the CC family in which the two cysteines are adjacent (MCPs, RANTES, Eotaxin, MIPs) (Prieschl et al., 1995; Bazan et al., 1997; Zlotnik et al., 1999). These proteins have no modifications other than two disulfide bonds and are multifunctional. Chemokines function in a very tightly regulated dose- and time-dependent manner, strongly suggesting that their actions are affected by the microenvironmental conditions (Dunleavy and Couchman, 1995; Gharaee-Kermani et al., 1996; Rennekampff et al., 1997; Young et al., 1997).

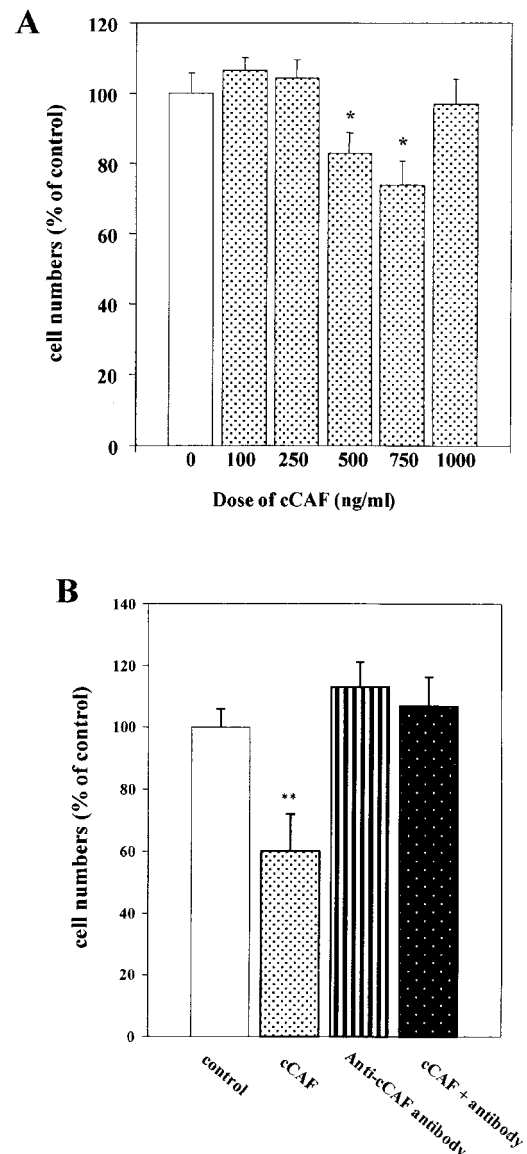
The first evidence that chemokines are associated with healing was reported in 1990 when it was shown that the chemokine chicken chemotactic and angiogenic factor (cCAF) is overexpressed during wound healing (Martins-Green and Bissell, 1990). This chemokine is highly expressed in the first 24–48 h after injury and remains elevated for at least 16 d after wounding (Martins-Green and Bissell, 1990; Martins-Green et al., 1992; Martins-Green and Hanafusa, 1997). It is primarily expressed by the fibroblasts of the granulation tissue, especially where interstitial collagen is abundant and by the endothelial cells of microvessels of the granulation tissue (Martins-Green and Bissell 1990; Martins-Green et al., 1996).

In the chicken chorioallantoic membrane (CAM) assay, at low doses cCAF is chemotactic for monocyte/macrophages and lymphocytes and after several days of exposure to this chemokine, the ectoderm of the CAM becomes thickened and a granulation-like tissue develops beneath the cCAF-containing pellet. In this granulation-like tissue, there is an increase in the amount of interstitial collagen and the fibroblasts in the mesoderm are consistently aligned with the collagen fibers and appear to cause tissue contraction (Martins-Green and Feugate, 1998). At high concentrations, however, cCAF stimulates blood vessel sprouting from the pre-existing vessels of the CAM (angiogenesis) in the absence of leukocyte chemotaxis (Martins-Green and Feugate, 1998).

Other CXC chemokines have also been associated with wound-healing events. For example, in burn wounds, gro $\alpha$ /MGSA is expressed by keratinocytes as they differentiate after reepithelialization of the wound. Furthermore, CXCR2, the receptor for MGSA, is present in migrating and proliferating keratinocytes (Nanney et al., 1995; Rennekampff et al., 1997). In the granulation tissue, MGSA expression is associated with fibroblasts, smooth muscle cells/myofibroblasts, and a subpopulation of macrophages (Nanney et al., 1995). This pattern of expression strongly suggests a role of this chemokine in healing of burn wounds. It also has been shown that in transgenic mice expressing IP-10, a CXC chemokine that inhibits angiogenesis, wounds heal poorly and exhibit defects in development of the granulation tissue (Luster et al., 1998).

It is becoming increasingly more evident that chemokines are expressed at the sites of injury and that they affect processes involving proper development of the granulation tissue. Fibroblasts are critical participants in the development of this healing tissue and they also express high levels of chemokines upon stimulation by stress-inducing agents such as those released upon wounding. Despite this correlative evidence, little is known about how these small cyto-

kines affect wound fibroblast function. cCAF is highly expressed by the fibroblasts of healing tissue, stimulates the formation of granulation-like tissue in the CAM, and is highly homologous to several human chemokines (Stoeckle and Barker, 1990). Therefore, we investigated the effects of this chemokine on proliferation and differentiation of fibroblasts, both important processes during granulation tissue development. We find that cCAF stimulates fibroblasts to differentiate into myofibroblasts and accelerates wound closure *in vivo*.



**Figure 1. The effects of cCAF on fibroblast growth.** (A) Primary connective tissue embryonic fibroblasts were treated for 3 d with increasing doses of cCAF. Treatment was applied every 24 h. cCAF suppresses fibroblast proliferation in a dose-dependent pattern characteristic of most chemokine functions. Maximum suppression was observed at a dose of 750 ng/ml. (B) Inhibition of cCAF function with an antibody specific to this chemokine abrogates this suppression of growth. Results shown are representative of several independent experiments. Double asterisk indicates  $P < 0.01$ ; Asterisk indicates  $P < 0.05$ .

## Results

### Effects of cCAF on fibroblast growth

To determine the effects of cCAF on fibroblast growth, primary chicken embryonic connective tissue fibroblasts were cultured in the presence of 2% donor calf serum. Embryonic fibroblasts behave much like wound fibroblasts (Brown et al., 1993), and the presence of a small amount of serum in the medium mimics the conditions of the wound, where serum factors are abundant. Because these are primary fibroblasts isolated weekly from different embryos, rather than cell lines, there are genetic variations and therefore differences in the levels of response to the various treatments. As a consequence, internal controls were always included and results from different batches of cells were not averaged. Therefore, all figures depict a representative experiment out of several performed for each type of experiment.

When fibroblasts are cultured in the presence of serum, cCAF suppresses proliferation of these cells by 25% compared with untreated cells (Fig. 1;  $P < 0.01$ ). This effect is dose dependent, with the greatest suppression occurring at 500–750 ng cCAF/ml medium (Fig. 1 A), and at 2 d after plating (unpublished data). It is commonly observed that chemokines cause their effects only in a narrow range of concentrations; at concentrations higher than the optimal dose, their receptors are very quickly desensitized and/or downregulated (e.g., Zlotnik et al., 1999; Murdoch and Finn, 2000). Although chemokine concentrations of  $10^{-2}$ – $10$  ng/ml can chemoattract and activate leukocytes, chemokines acting on other cell types such as endothelial cells, smooth muscle cells and fibroblasts require concentrations in the range of  $10^2$  ng/ml (Gupta and Singh, 1994; Gharaee-Kermani et al., 1996; Luo et al., 1996). This is within physiological range; wound fluid from burn patients has MGSA concentrations of  $10^2$ – $10^3$  ng/ml at 6–7 d after injury (Rennekampff et al., 1997). Experiments using an antibody specific for cCAF showed that this antibody abrogates the effects of this chemokine on proliferation (Fig. 1 B).

Although the decrease in growth induced by cCAF is significant, it is not large. Therefore, we examined the possibility that this reduction in cell numbers was due to increased cell death. Cells grown under the same conditions as above were prepared to detect apoptosis by determining whether DNA laddering occurs. Both untreated fibroblasts and those treated with cCAF had intact DNA (Fig. 2 A), showing that this chemokine did not induce cells to undergo apoptosis. Similar studies performed to detect whether cCAF induced necrotic cell death using trypan blue showed that this chemokine does not cause cells to undergo necrosis (Fig. 2 B).

We have shown previously that the COOH-terminal peptide (28 amino acids) of cCAF is by itself angiogenic in vivo (Martins-Green and Feugate, 1998; Martins-Green and Kelly, 1998). Therefore, we tested the possibility that these effects of cCAF on fibroblast growth are also mediated by the COOH terminus of the molecule. Cells were treated with the COOH-terminal peptide following the same regimen of treatment as for cCAF; we found that this peptide did not cause a decrease in cell numbers (Fig. 3). We then turned to the NH<sub>2</sub> terminus of the molecule because it has been shown for several chemokines that their chemotactic properties for leukocytes require this portion of the protein (Clark-Lewis et

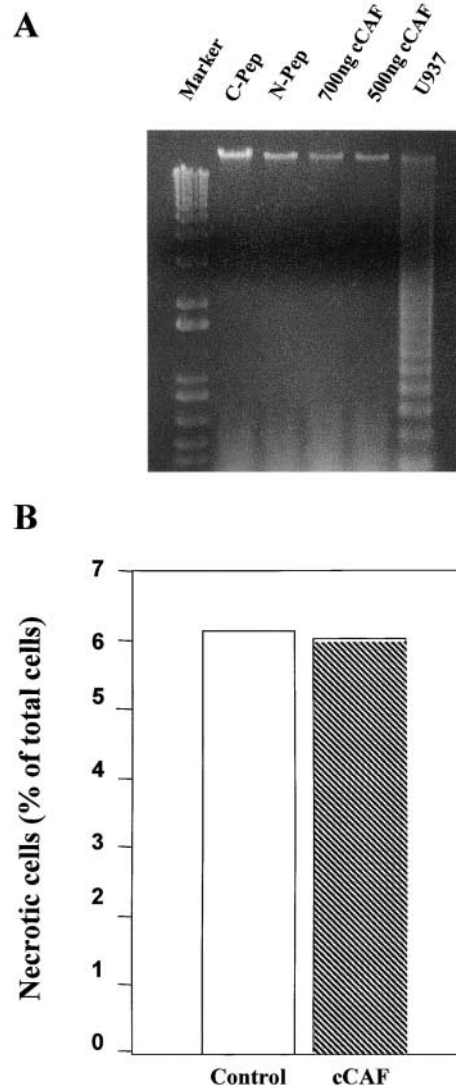
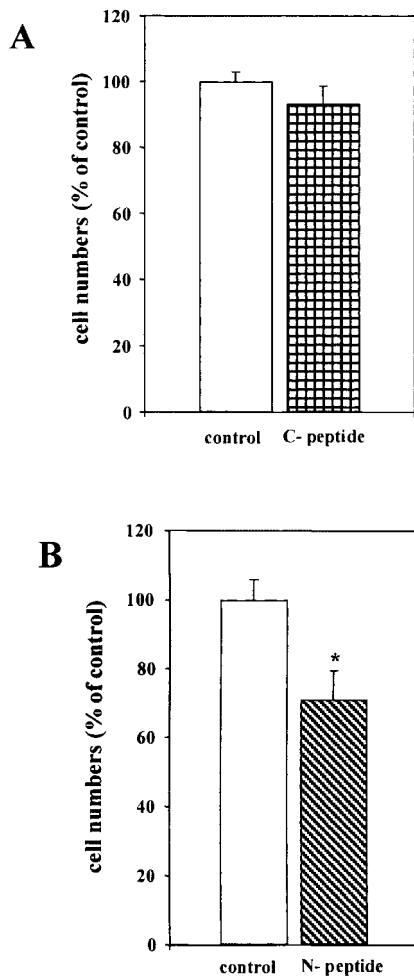


Figure 2. **The effects of cCAF on cell death.** (A) To detect cCAF-induced apoptosis we used the DNA Laddering Detection system from Roche Biochem. Fibroblasts were plated, treated as described in Materials and methods, the DNA was prepared as suggested by the manufacturer, and agarose gel electrophoresis was performed. No DNA laddering was detected in cells treated with cCAF, N-peptide, or C-peptide. The positive control for DNA laddering provided by the manufacturer consisted of U937 cells treated for 3 h with camptothecin (4  $\mu$ g/ml). (B) Cells incubated with trypan blue, which stains necrotic cells blue, showed that cCAF does not stimulate cell death by necrosis. The results shown represent one of three experiments.

al., 1995; Strieter et al., 1995; Weber et al., 1996; Baggiolini et al., 1997; Martins-Green and Feugate, 1998). Treatment of fibroblasts with the first 15 amino acids of the NH<sub>2</sub> terminus (N-peptide) resulted in a decrease in growth similar to that induced by cCAF (Fig. 3). Furthermore, the dose- and time-dependent pattern of suppression of growth induced by the N-peptide was the same as that of the whole cCAF molecule, albeit at a higher molar concentration (unpublished data).

### cCAF stimulates $\alpha$ -SMA expression

The results described above show that the decrease in cell numbers induced by cCAF is specific and not due to cell



**Figure 3. Effects of the C- and N-peptides of the cCAF protein on fibroblast growth.** Treatment with the C-peptide (28 amino acids) did not suppress the growth of connective tissue fibroblasts, whereas treatment with the N-peptide (15 amino acids) suppresses the growth of fibroblasts to a level similar to that caused by the whole cCAF molecule. Results shown are representative of three independent experiments. Asterisk indicates  $P < 0.05$ .

death, but that the effect is small and requires 2–3 d to be detected, even with repeated applications of cCAF. This could mean that cCAF plays a role in triggering fibroblasts to slow down growth and develop a differentiated phenotype with a consequent decrease in cell division. Therefore, we investigated the possibility that cCAF stimulates differentiation of fibroblasts into myofibroblasts, cells that play an important role in wound closure and proliferate at a slower rate than normal fibroblasts (Masur et al., 1996; Nedelec et al., 1998; Dimitrijevic-Bussod et al., 1999; Khouw et al., 1999). Immunolabeling of cCAF-treated fibroblasts with an antibody specific for  $\alpha$ -SMA, a marker for myofibroblasts, revealed the presence of many cells showing intense staining for fiber bundles, whereas control cells showed fewer and less brightly stained fibers (Fig. 4, A and B). As was found for the growth studies, treatment with the  $\text{NH}_2$ -terminal peptide stimulated  $\alpha$ -SMA production, whereas the COOH-terminal peptide did not (Fig. 4, C and D). Immunoblot analysis of cell extracts using this antibody showed increased levels of  $\alpha$ -SMA in cells treated with cCAF or the

N-peptide compared with cells treated with the C-peptide and control cells (Fig. 4 E). Inhibition of cCAF or N-peptide function by treatment of the cells in the presence of the cCAF antibody abrogated the increase in  $\alpha$ -SMA expression (Fig. 4, F and G).

Although  $\alpha$ -SMA is considered the characteristic marker for myofibroblasts, vimentin, desmin, and myosin heavy chain, as well the muscle proteins myoD and myogenin, are sometimes expressed by myofibroblasts (Skalli et al., 1989; Mayer and Leinwand, 1997). Using antibodies recognizing the chicken proteins, we immunoblotted cell extracts from cCAF and N-peptide-treated fibroblasts. There was no detectable desmin, myosin heavy chain, or myoD present in either control or treated cells (unpublished data). However, vimentin levels increased with cCAF or N-peptide treatment in a manner similar to that observed for  $\alpha$ -SMA (Fig. 5). Although desmin and the sarcomeric proteins myoD and MHC are expressed in some myofibroblasts, their presence or absence depends on the origin of the myofibroblasts, whereas most myofibroblasts express both vimentin and  $\alpha$ -SMA (Gabbiani, 1994).

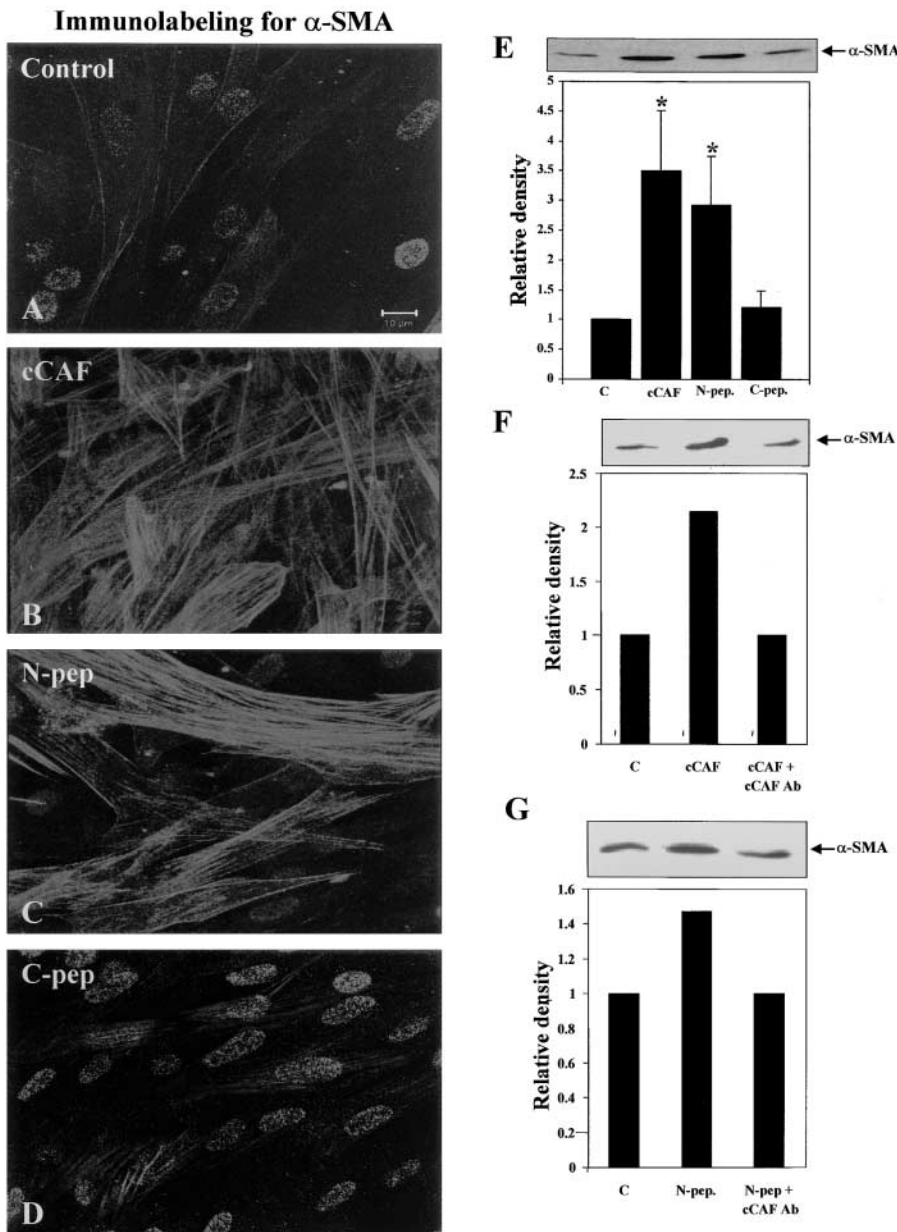
To determine whether this increase in  $\alpha$ -SMA and vimentin results in a functional phenotype, we examined the effect of cCAF on contraction of fibroblast-seeded collagen gels. Fibroblasts plated on collagen often grow in clusters, but cellular clusters in plates treated with cCAF or N-peptide were more contracted than clusters in control cells (Fig. 6, A–C). When collagen gels were released after 4–5 d of treatment, cCAF or N-peptide-treated cells contracted the gels more tightly than did untreated cells (Fig. 6, D–F). Immunolabeling of sections prepared from collagen gels with the antibody to  $\alpha$ -SMA showed that treated gels contain more cells with fibers staining for  $\alpha$ -SMA than control gels (Fig. 6, G–I). Fig. 6 J shows a quantitative analysis of cCAF- and of N-peptide-induced collagen gel contraction. To demonstrate that this contraction is the result of an increase in  $\alpha$ -SMA expression, we treated the cells with phosphorothioate antisense oligodeoxynucleotides (ODN) to inhibit production of  $\alpha$ -SMA. Antisense ODN strongly decreased the ability of the cells treated with cCAF or the N-peptide to contract the collagen gels, but did not have an effect on the contractility of cells treated with the COOH terminus (Fig. 7 A). Detection of  $\alpha$ -SMA in these gels shows that the levels of this protein in the gels treated with antisense oligonucleotides is similar to those of the control, whereas the levels in the gels treated with cCAF or the N-peptide alone or in the presence of sense oligonucleotides, have higher levels of  $\alpha$ -SMA (Fig. 7 B).

To determine whether cCAF directly stimulates  $\alpha$ -SMA mRNA expression, we used reverse transcription (RT)-PCR with specific primers to this molecule to examine the effects of cCAF on mRNA levels. This chemokine causes an increase in  $\alpha$ -SMA mRNA levels within 1 h of treatment, and mRNA levels remain elevated for more than 12 h after treatment (Fig. 8).

#### cCAF-induced effects on wound closure

In wounded tissue, myofibroblast differentiation contributes to closing of wounds (Nedelec et al., 1998). Our finding that cCAF stimulates fibroblasts to acquire myofibroblastic



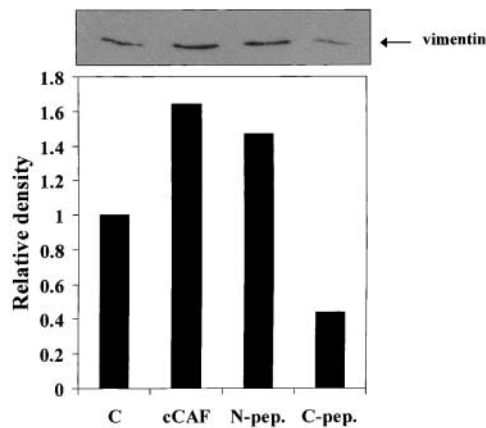


**Figure 4. Effect of cCAF and the NH<sub>2</sub> terminus on  $\alpha$ -SMA expression.** (A) Untreated embryonic connective tissue fibroblasts were immunolabeled for  $\alpha$ -SMA, a marker for myofibroblast differentiation. Some fibroblasts show a small amount of staining for this protein, which is characteristic of fibroblasts in culture. (B) Cultures treated with cCAF or (C) with the N-peptide for 3 d show that more fibroblasts are stained for  $\alpha$ -SMA, that more fibrils are present in the cells, and that the staining is more intense than for untreated cells or those treated with the C-peptide (D). (E) Immunoblot analysis for  $\alpha$ -SMA to quantify the results observed in (A–D). All lanes in the immunoblots contain equal amounts of total protein, as measured by the DC protein assay (Bio-Rad Laboratories). Cells treated with 750 ng/ml of cCAF or of the N-peptide show much higher levels of  $\alpha$ -SMA when compared with untreated or C-peptide-treated cells.  $n = 4$  and asterisk indicates  $P < 0.05$ . Inhibition of cCAF (F) or N-peptide (G) with an anti-cCAF antibody blocks the increase in  $\alpha$ -SMA expression. Results shown in E, F, and G are representative of three or more experiments.

phenotype and function in culture led us to determine whether cCAF affects wound closure in vivo. Full thickness and same size excision wounds were made on the underside of wings of 2-wk-old chicks, and for each bird one wing was treated with vehicle (H<sub>2</sub>O) and the other with 1  $\mu$ g of cCAF every other day. The bandages were removed at days 3, 5, and 7, the wounds were photographed, dressed with clean bandages, and treatments applied. For each wound, rate of wound closure was examined by taking digital photographs of the same wound through time and then determining the areas of the wounds from the photographs using NIH Image. Fig. 9, A and C, show a wounded wing before and after 3 d of treatment with vehicle, whereas Figs. 9, B and D, show the contralateral wing of the same bird, treated with cCAF at the same times. Fig. 9 E shows the results of analyses and comparison of the wound areas of several birds over time. On day 3, the cCAF-treated wounds had closed to a significantly greater degree than the vehicle-treated

wounds. At day 5, the closure of the control wounds was still less than that of the experimental wounds, but closure in the controls may have accelerated during this period. Although cCAF-treated wounds close faster, by day 7 both control and treated wounds were completely closed. Wounds treated with the N-peptide also showed accelerated wound closure during days 0–3, albeit less strongly than with the whole cCAF molecule (Fig. 9 F).

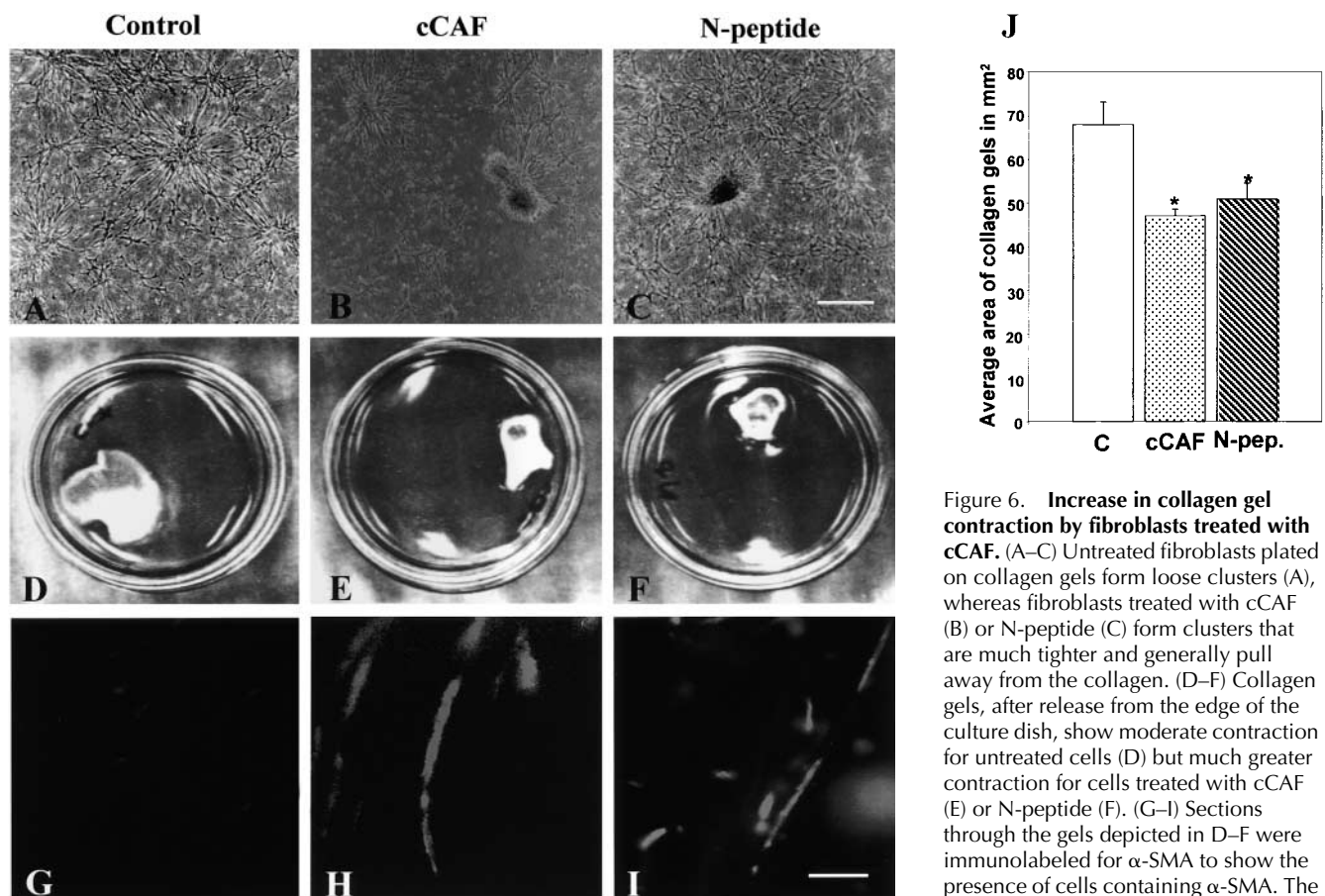
Sections through the wounds show that the granulation tissue of cCAF-treated wounds appears more dense earlier in wound healing (days 3 and 5) than the tissue of the wounds treated with vehicle (Fig. 10, A–D). In particular, the granulation tissue of the control wounds at day 5 (Fig. 10 C) resembles that of the treated on day 3 (Fig. 10 B). By day 7, the cCAF-treated wounds appear to be already remodeling, whereas those treated with vehicle looked much like the granulation tissue of cCAF-treated wounds at 5 d (Fig. 10, E and F). It is known that wound



**Figure 5. Effect of cCAF and the NH<sub>2</sub> terminus on vimentin expression.** Immunoblot analysis for vimentin, another marker for myofibroblasts. Cells treated with 750 ng/ml of cCAF or of the N-peptide show higher levels of vimentin than do untreated or C-peptide-treated cells. All lanes contain equal amounts of total protein, as measured by the DC protein assay (Bio-Rad Laboratories).

contraction causes the healing tissue to become more dense and compact, therefore our findings strongly suggest that cCAF stimulates early wound contraction and closure.

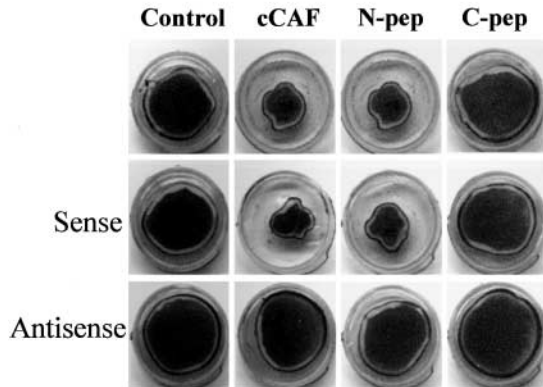
To test whether this acceleration in wound closure was due to an increase in the number of myofibroblasts in the granulation tissue, we immunostained vehicle-treated and cCAF-treated wounds for  $\alpha$ -SMA (Fig. 11). At day 3, the granulation tissue of the cCAF-treated wounds has many more cells that label for  $\alpha$ -SMA (Fig. 11 B) than does the tissue of vehicle-treated wounds (Fig. 11 A). On day 5, there continue to be many more cells staining for  $\alpha$ -SMA in the cCAF-treated wounds (Fig. 11 D) than in the control tissue (Fig. 11 C). By day 7, this difference has greatly decreased; the treated and untreated wounds have similar staining for myofibroblasts (Fig. 11, E and F). Quantification of the number of cells labeling for  $\alpha$ -SMA in the wounds shows that there are significantly more myofibroblasts in the cCAF-treated wounds at days 3 and 5, but the difference is no longer significant by day 7 (Fig. 11 G). The amount of  $\alpha$ -SMA in the wounds was not quantified by Western blotting because wounds treated with cCAF contain many more new blood vessels that are surrounded by cells that express  $\alpha$ -SMA. Whereas it is easy to distinguish the blood vessel-associated cells that contain  $\alpha$ -SMA from myofibroblasts in the granulation tissue by microscopy, we would not be able to make that distinction by immunoblot analysis. The earlier appearance of  $\alpha$ -SMA-staining cells in wounds treated with cCAF suggests that the acceleration of wound contraction by cCAF is due to an increase in myofibroblasts at the early stages of wound repair.



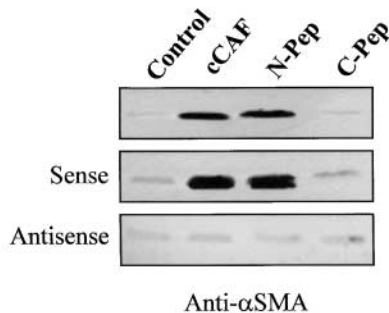
**Figure 6. Increase in collagen gel contraction by fibroblasts treated with cCAF.** (A–C) Untreated fibroblasts plated on collagen gels form loose clusters (A), whereas fibroblasts treated with cCAF (B) or N-peptide (C) form clusters that are much tighter and generally pull away from the collagen. (D–F) Collagen gels, after release from the edge of the culture dish, show moderate contraction for untreated cells (D) but much greater contraction for cells treated with cCAF (E) or N-peptide (F). (G–I) Sections through the gels depicted in D–F were immunolabeled for  $\alpha$ -SMA to show the presence of cells containing  $\alpha$ -SMA. The staining is much less intense in untreated

cells (G) than in cells treated with cCAF (H) or N-peptide (I). (J) Contracted gels were photographed and their areas were measured and analyzed using NIH Image. Treatments with both cCAF and N-peptide stimulated fibroblasts to contract gels more efficiently than did untreated cells. Asterisk indicates  $P < 0.05$ . Bars, 100  $\mu$ m.

A



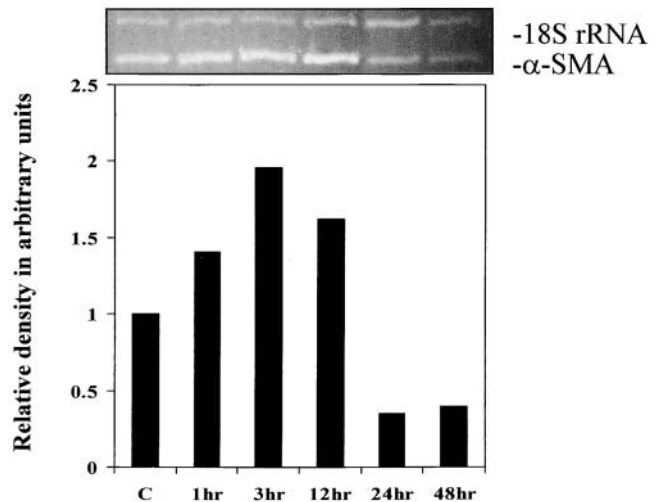
B



**Figure 7. Inhibition of  $\alpha$ -SMA expression using oligonucleotides.** (A) Phosphorothioate antisense ODN were used to inhibit  $\alpha$ SMA expression. The sequence of ODN is provided in Materials and methods. When preparing the collagen gels, 2  $\mu$ M of antisense or sense ODN were incorporated into the gel before the seeding of CEFs. The treatments to induce gel contraction were performed as in Fig. 6, A–F, except that 2  $\mu$ M of antisense or sense ODN was added each time the chemokine or peptide was applied. The ODN strongly inhibited gel contraction stimulated by cCAF or the N-peptide. (B) Immunoblot analysis of  $\alpha$ SMA production in the gels from the experiments in A.

## Discussion

The work presented here shows that the CXC chemokine, cCAF, when applied repeatedly, suppresses fibroblast growth to  $\sim 75\%$  of untreated cells, in a time- and dose-dependent manner. This decrease in growth is not due to cell death but rather it correlates with cCAF-induced expression of the myofibroblast marker,  $\alpha$ -SMA. Although the decrease in proliferation takes time to be detected,  $\alpha$ -SMA expression levels rise rapidly following cCAF treatment, strongly suggesting that this chemokine stimulates myofibroblast differentiation directly. The expression of  $\alpha$ -SMA in cCAF-treated cells results in an increase in the ability of the fibroblasts to contract collagen gels. Specific inhibition of  $\alpha$ -SMA expression resulted in abrogation of contraction. All of these effects are specific and can be accomplished by the  $\text{NH}_2$ -terminal peptide of the cCAF molecule by itself. Furthermore, in vivo, when applied to excision wounds, this chemokine accelerates wound closure and the granulation



**Figure 8. Stimulation of  $\alpha$ -SMA expression in cells treated with cCAF.** Fibroblasts treated with cCAF show rapid increase of  $\alpha$ -SMA mRNA, peaking at 3 h and declining by 24 h. Total RNA extracted using TRIzol reagent and RT-PCR was performed as described in Materials and methods. A fragment of the 18S rRNA was used as control to normalize the amount of  $\alpha$ -SMA.

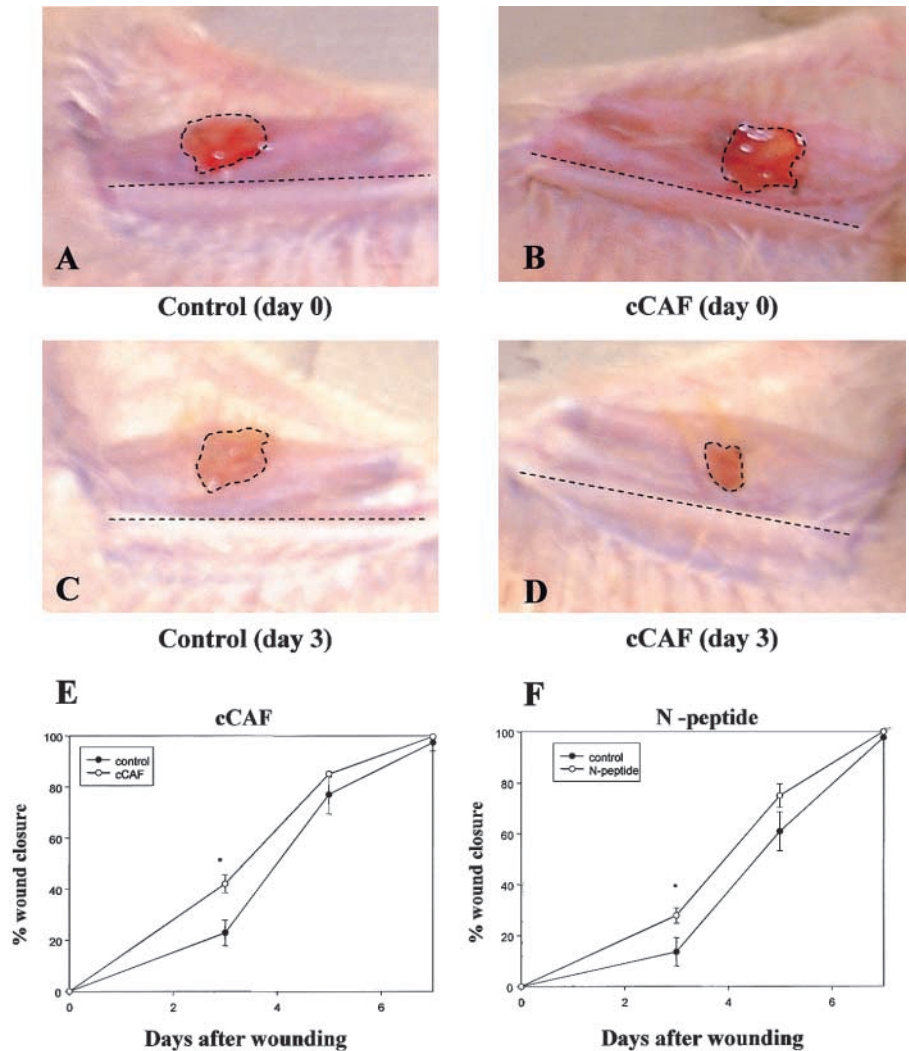
tissue becomes more dense early in wound healing. This correlates with an increase in the number of myofibroblasts in the tissue. These results indicate that cCAF stimulates fibroblasts to differentiate into myofibroblasts and strongly suggest that this function may be responsible for the more effective wound closure induced by this chemokine.

Although little is known about chemokines and wound closure, expression of cCAF, IL-8, and MGSA is elevated until wound closure and then decreases to low but still elevated levels during granulation tissue formation (Martins-Green and Bissell, 1990; Engelhardt et al., 1998). In addition, knockout mice for CXCR2, a receptor for IL-8 and MGSA, exhibit delayed wound closure (Devalaraja et al., 2000). The results presented here shine light into these findings in vivo. Our observations suggesting that cCAF stimulates  $\alpha$ -SMA expression directly, that this elevation in expression leads to increased contraction of collagen gels and to more rapid wound contraction and closure, indicate that chemokines potentially play significant roles in formation of the granulation tissue of wounds. Furthermore, the small (15 amino acids)  $\text{NH}_2$ -terminal peptide of the cCAF molecule has the same effects, suggesting that similar behavior in a human chemokine could be a promising target in designing drugs that affect the differentiation of myofibroblasts.

It has been shown that myofibroblasts can differentiate from fibroblasts when these cells are exposed to TGF $\beta$ 1 in culture (Desmouliere et al., 1993) and that, in vivo, this growth factor directly stimulates myofibroblast differentiation (Serini and Gabbiani, 1999). Furthermore, most previously known stimulators of myofibroblast differentiation appear to act indirectly through TGF $\beta$ 1 (Serini and Gabbiani, 1999). Whether cCAF can stimulate  $\alpha$ -SMA independently of TGF $\beta$  is not known. However, the fact that this chemokine stimulates  $\alpha$ -SMA expression shortly after treatment is initiated, and that it stimulates signals through G-protein-coupled receptors, whereas TGF $\beta$ 1 elicits its effects through



**Figure 9. Accelerated closure of wounds treated with cCAF or with N-peptide.** Excision wounds were made on the underside of chick wings and then treated every other day with vehicle ( $H_2O$ ) or  $1 \mu g$  cCAF. The dashed lines trace the edges of the wounds. The underlying muscle can be seen within the wounds. Control wings (A and C) show less wound closure at day 3 than cCAF-treated wounds (B and D). Dashed straight lines mark the length of the forewings to facilitate evaluation of the size of the wounds. (E) Average percent wound closure in eight birds for cCAF or vehicle treatment. (F) Average percent wound closure of eight birds/treatment with N-peptide or vehicle. Areas of the wounds were measured on digital photographs using NIH Image software. Percentage wound closure was calculated by comparing the area of the closing wound to the area of the same wound on day 0. At day 3,  $P < 0.05$  for both treatments.



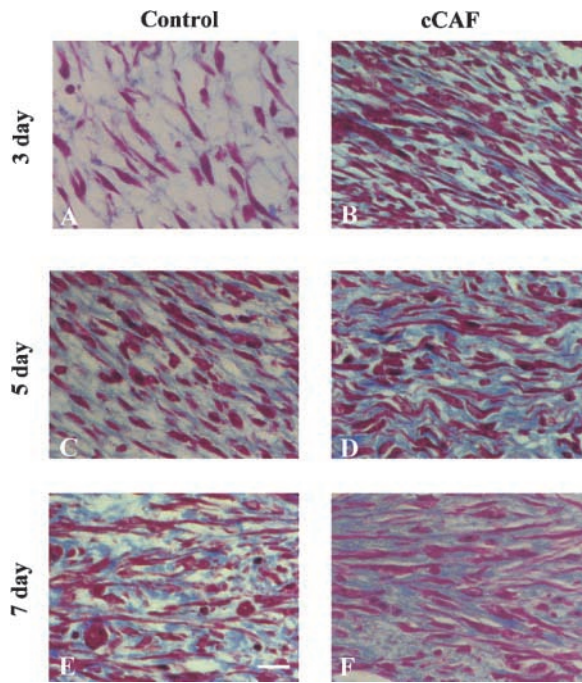
serine kinase receptors, suggests that they stimulate different signal transduction pathways to activate  $\alpha$ -SMA expression. Nevertheless, it is possible that these signal transduction pathways share common downstream signaling molecules. We are currently investigating the molecular mechanisms of cCAF-induced  $\alpha$ -SMA production, whether the human homologues of cCAF function in the same manner, and how they relate to the signaling mechanisms stimulated by TGF $\beta$ .

Cells containing  $\alpha$ -SMA (myofibroblasts, smooth muscle cells, and pericytes) play important functions in a variety of processes involved in wound healing, vasculogenesis/angiogenesis, and pathological conditions, especially in diseases that are characterized by excess scarring. In wound healing, myofibroblasts are particularly important in wound closure and contraction. For example, lack of myofibroblasts after corneal surgery leads to corneal flattening and widening of the wound, whereas stimulation of myofibroblast differentiation in noncontractile fetal wounds leads to contraction of the wounds (Jester et al., 1999; Lanning et al., 2000). In disease states characterized by an accumulation of myofibroblasts, such as pulmonary fibrosis and scleroderma, myofibroblasts are thought to contribute significantly to the pathology of the disease, primarily because myofibroblasts tend to be highly fibrogenic (Powell et al., 1999). In addition,

myofibroblasts are present in the stroma of many tumors and appear to be important for the survival of these tumors (Coffin et al., 1998; Schürch et al., 1998).

In addition to stimulating wound closure through the differentiation of myofibroblasts, cCAF may also be acting to increase the stability of new blood vessels in the granulation tissue. Smooth muscle cells of the new vasculature are known to differentiate from mesenchymal cells in response to signals from the endothelial cells (e.g., Carmeliet, 2000). These smooth muscle cells are essential for vascular maturation in connective tissue (Carmeliet, 2000). CXC chemokines are produced by the endothelial cells and fibroblasts of the connective tissue and promote angiogenesis (Martins-Green and Bissell, 1990; Martins-Green et al., 1991, 1992; Martins-Green and Feugate, 1998; Belperio et al., 2000). These chemokines are known to affect endothelial cell migration, but part of their role in the formation of new blood vessels may be in stimulating fibroblasts to acquire  $\alpha$ -SMA and become the smooth muscle cells that stabilize the newly formed vasculature (unpublished data).

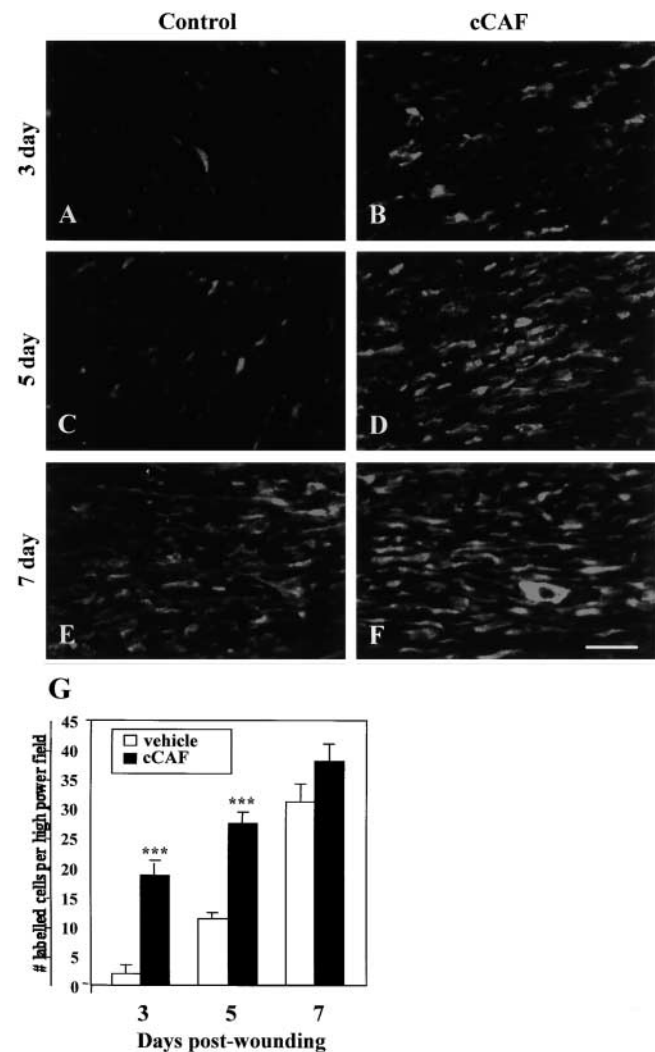
Because myofibroblast accumulation is prominent and high levels of chemokines are present in conditions characterized by excessive scarring, such as keloids, scleroderma, and pulmonary fibrosis (Zhang et al., 1996; Nedelec et al.,



**Figure 10. Histology of excision wounds treated with cCAF.** Paraffin-embedded sections of excision wounds treated every other day with vehicle or 1  $\mu$ g cCAF were stained with Masson Trichrome. At 3 d after wounding, control wounds (A) have much less dense granulation tissue than those treated with cCAF (B). By day 5, the granulation tissue of the control wounds (C) resembles the tissue of the treated wounds at day 3 (B). The cCAF-treated wounds continue to have denser repair tissue than the control wounds on day 5 (D). By day 7, the difference between the control wounds (E) and cCAF-treated wounds (F) is greatly diminished. Bar, 25  $\mu$ m.

1998), it is possible that chemokines may participate in such diseases by stimulating myofibroblast differentiation. For example, levels of MCP-1, IL-8, and MIP-1 $\alpha$  are high in pulmonary fibrosis (Keane et al., 1997; Hasegawa et al., 1999). These chemokines are also elevated in sclerotic tissue (Kadono et al., 1998; Hasegawa et al., 1999). Myofibroblasts in keloids express MGSA, whereas the cells of normal scars do not (Nirodi et al., 2000). Our results suggest that some of the problems in these conditions may be due to the high levels of chemokines, leading to an increase in myofibroblast numbers, excess deposition of matrix molecules, and contraction of the tissue.

Controlling the differentiation of myofibroblasts could mitigate the effects of fibrotic and other diseases. CXC chemokines could be suitable for this purpose because they are not constitutively expressed and do not have the broad-ranging effects that TGF $\beta$ 1 does. In addition, chemokines are very small molecules with no modification other than disulfide bonds, therefore they can be produced using recombinant approaches without much difficulty and they bind to 7-transmembrane receptors, which are highly amenable to pharmacological manipulations. As a consequence, once the mode of action of these proteins on  $\alpha$ -SMA expression is deciphered, they or their antagonists could be used to modulate the presence of myofibroblasts in both disease states and in abnormal wound healing. Furthermore, the ability of the 15 amino acid NH<sub>2</sub>-terminal peptide to stimu-



**Figure 11. Immunostaining for  $\alpha$ -SMA in the granulation tissue of excision wounds treated with cCAF.** Paraffin-embedded sections of excision wounds treated every other day with vehicle or 1  $\mu$ g cCAF were immunostained with an antibody against  $\alpha$ -SMA. At 3 d after wounding, control wounds (A) show almost no cells labeling for  $\alpha$ -SMA, whereas those treated with cCAF show already a significant number of cells labeling for  $\alpha$ -SMA (B). At day 5, both controls (C) and cCAF-treated wounds (D) show more cells expressing  $\alpha$ -SMA, with cCAF-treated wounds showing a much greater increase. By day 7, cCAF-treated wounds (F) continue to have more labeled cells than do control wounds (E), but the difference is greatly diminished. (G) Quantitation of the data in A–F (see Materials and methods for quantitation procedures). Triple asterisk indicates  $P < 0.001$ . Bar, 88  $\mu$ m.

late effects similar to those stimulated by the whole cCAF molecule strongly suggests that the peptide itself or peptide mimetics could be used for treatment of wounds with impaired closure.

In conclusion, a major role of cCAF in the granulation tissue development may be the stimulation of proper wound closure through the stimulation of myofibroblast differentiation. This is a previously unknown function for chemokines and it could represent a novel mechanism for the induction of myofibroblast differentiation. In addition, our results may explain why chemokines contribute to the

pathology of fibrotic diseases in which myofibroblasts play a significant part.

## Materials and methods

### Materials

All tissue culture media and materials were purchased from GIBCO BRL. cCAF was either purified as described in Martins-Green and Feugate (1998) or synthesized by Gryphon Sciences. The 16 amino acid NH<sub>2</sub>-terminal peptide was also synthesized by Gryphon Sciences. The 28 amino acid COOH-terminal peptide was synthesized by Milligen Bioscience. Antibodies used were: anti- $\alpha$ -SMA (Sigma-Aldrich); anti-TGF $\beta$  1,2,3 (R&D Systems); antivimentin, antidesmin, antimyosin heavy chain (Hybridoma Bank); anti-myosin D (Santa Cruz Biotechnology, Inc.); anti-mouse horseradish peroxidase, anti-mouse Texas red (Amersham Pharmacia Biotech); anti-mouse Alexa (Molecular Probes); and anti-mouse FITC (Dako). The anti-cCAF rabbit serum was prepared by Robert Sargeant (Ramona, CA). Other materials used include: nuclear staining To-Pro3 (Molecular Probes), ECL reagents (Amersham Pharmacia Biotech), Vectashield mounting medium (Vector Laboratories), DC protein assay kit (Bio-Rad Laboratories), TRIzol reagent (GIBCO BRL), Vitrogen 100 collagen (Cohesion Technologies), Biocclusive bandage (Johnson and Johnson Medical); and Classic 18S rRNA primers (Ambion). TGF $\beta$ 1 was a gift from A. Roberts (National Institutes of Health, Bethesda, MD).

### Cell cultures

Primary chicken embryonic connective tissue fibroblasts (CEFs) were cultured as described previously in Martins-Green and Feugate (1998). Briefly, these fibroblasts were isolated from 10-d-old chicken embryo body walls, plated at  $6 \times 10^6$  cells/100-mm plate and cultured for 4 d in 199 medium with 5% donor calf serum, 1% chick serum, and 0.3% tryptose phosphate broth. These cultures were passaged once (secondary cultures); the cells were plated at  $0.4 \times 10^6$  cells/35-mm plate in 199/tryptose phosphate broth and 2% donor calf serum.

### Fibroblast growth assay

Secondary fibroblasts were plated at  $0.4 \times 10^6$  cells/35-mm plate and cCAF was added to experimental plates. For each experiment, plating efficiency was determined by trypsinizing plates 4 h after plating. Cells were counted with a Coulter particle counter to confirm even plating of cells. The media was replaced approximately every 16 h with 1 ml of serum-free 199 medium containing 0.3% tryptose phosphate broth and 2% donor calf serum, and 100–1,000 ng cCAF (9–90 nM) or C- or N-peptide (64–640 nM) was added to the experimental plates, whereas control plates contained media only. On day 3, plates were trypsinized to remove all cells and cells were counted using a Coulter counter. To test for specificity, anti-cCAF rabbit serum was preincubated with cCAF (3  $\mu$ l serum/750 ng cCAF [68 nM] or N-peptide [480 nM]) for 1 h at room temperature before being added to cells.

### Trypan blue staining

The supernatant of treated and untreated CEFs was collected and the cells centrifuged to a pellet and resuspended in a small volume of medium. 1% trypan blue was added to a final concentration of 0.5% trypan blue. Cells were stained for 5 min and then counted in a hemocytometer. Necrotic cells stained blue.

### Apoptosis assay

To detect cCAF-induced apoptosis we used the DNA Laddering Detection System from Roche Biochem. Fibroblasts were plated, treated as described above, and the DNA was prepared as suggested by the manufacturer. Briefly, at the end of each treatment, cells were trypsinized, pelleted, resuspended in binding buffer, and incubated for 10 min at room temperature. After this incubation period, isopropanol was added and the preparation vortexed and passed through a filter tube with glass binding fleece. The samples were then centrifuged for 1 min at 8,000 rpm, the flow through was discarded, and the DNA retained in the fleece was washed two times with wash buffer and then eluted with prewarmed elution buffer and used for agarose gel electrophoresis.

### Immunostaining for $\alpha$ -SMA

Plates of fibroblasts were treated with cCAF as described previously. After 4 d, the cells were rinsed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.15% Triton X-100, and incubated with PBS containing

0.1 M glycine for 10 min. Cells were blocked for 30 min with 10% goat serum in PBS, incubated with mouse anti- $\alpha$ -SMA to a final IgG concentration of 20  $\mu$ g/ml in 1% BSA/PBS for 1 h at room temperature, and washed three times with 0.1% BSA/PBS for 10 min each. The cells were then incubated in To-Pro3 (1:1,000) and goat anti-mouse FITC or sheep anti-mouse Texas red (1:100) in 1% BSA/PBS for 1 h at room temperature, washed three times for 10 min with 0.1% BSA in PBS, and mounted with Vectashield. Confocal fluorescence microscopy was performed on a ZEISS LSM510. Collagen gels were rinsed with PBS, fixed in 4% paraformaldehyde for 2 h, washed three times 30 min each with PBS, and incubated for an additional 30 min in PBS containing 0.1 M glycine. This treatment was followed by incubation at 4°C O/N in 15% sucrose and then incubation under the same conditions in 30% sucrose. After a brief rinse with PBS, the gels were frozen in OCT, sections were prepared and collected on gelatin-coated slides, rinsed with PBS, fixed in 4% paraformaldehyde for 10 min, and then incubated again in PBS containing 0.1 M glycine for 10 min. This was followed by blocking for 30 min with 10% goat serum in PBS, incubation in primary and secondary antibody as described above, and mounting with Vectashield.

### Immunoblotting

Plates of fibroblasts were treated as described previously with 750 ng/ml cCAF or 2.5 ng/ml TGF $\beta$ . To block TGF $\beta$  or cCAF activity, anti-cCAF (3  $\mu$ l) or anti-TGF $\alpha$  (1  $\mu$ l) antibody was preincubated in 1 ml media for 1 h at room temperature before being added to cells. After 4 d of treatment, protein extracts were prepared in 1 ml 150 mM RIPA buffer containing protease inhibitors. Protein concentrations were determined using the DC protein assay kit and samples were adjusted to contain equal amounts of protein. SDS-PAGE was performed on 7.5% separating Doucet gels (Doucet and Trifaro, 1988). Protein transfer to nitrocellulose was performed using a wet-transfer apparatus (Bio-Rad Laboratories) at 100 V for 45 min. The membranes were blocked for 1 h in 5% milk in TTBS and then incubated overnight at 4°C in anti- $\alpha$ -SMA (1:1,500), antivimentin (1:100), antidesmin (1:100), antimyosin heavy chain (1:100), or anti-myosin D (1:200) in 1% milk in TTBS. The membranes were washed three times for 20 min each with TTBS, incubated in anti-mouse HRP at 1:10,000 in 1% milk for 1 h, and washed as above; and the bands were visualized using the ECL.

### Collagen gel contraction

1.5 ml Vitrogen 100 collagen gels were made in 35-mm plates. Secondary fibroblasts were plated at  $0.4 \times 10^6$  cells/35-mm plate on top of the gels. After the cells had adhered to the collagen, cCAF was added to experimental plates. The medium was replaced approximately every 16 h with 1 ml of 199 supplemented with 0.3% tryptose phosphate broth and 2% donor calf serum, and 750 ng cCAF (68 nM) or N-peptide (480 nM) was added to the experimental plates. Control plates contained media only. After 4 d, gels were released. Treatment was continued for two more days and gels were photographed every 12 h. The photographs were used for evaluation of gel contraction by determining the area of the gel using NIH image analysis. For the experiments involving inhibition of  $\alpha$ -SMA expression, we used phosphorothioate antisense ODN, which was synthesized and HPLC purified by Sigma-Aldrich Genosys, to block the  $\alpha$ -SMA production. The sequence of ODN is specific for the 3'-untranslated region of  $\alpha$ -SMA (5'-CACAGTAATATGCTAAAAAGAC-3') as described previously (Ronnov-Jessen and Petersen, 1996; Nakajima et al., 1999). The sense strand of this ODN was used as control (5'-GTCTTTTATAGCATATTACTGTG-3'). When preparing the collagen gels, 2  $\mu$ M of antisense or sense ODN were incorporated into the gel before the seeding of CEFs. The treatments and measurement of gel contraction were performed as described above, except that 2  $\mu$ M of antisense or sense ODN were applied each time the chemokine or peptide was applied.

### RT-PCR

Total RNA for  $\alpha$ -SMA was extracted using TRIzol reagent from untreated fibroblasts, fibroblasts treated with 750 ng/ml cCAF, or fibroblasts treated for varying periods of time with cCAF. The RT-PCR procedure was performed using the Promega Access RT-PCR System, which is designed to finish RT and PCR in one tube and following the protocol recommended by Promega, except that 1.5 times the recommended amount of dNTP, reverse transcriptase, and Tfl DNA polymerase were used to ensure strong synthesis of 18S ribosomal RNA. The reaction conditions included: 1  $\mu$ g total RNA, first strand synthesis at 48°C for 45 min, then 95°C for 5 min to inactivate the reverse transcriptase, followed by DNA amplification at 95°C for 45 s, 58°C for 60 s, 68°C for 90 s for 40 cycles. Finally, 68°C for 7 min to extend the strands. 3  $\mu$ l of Quantum mRNA classic 18S primers (Ambion) were added to the reaction to produce the control band. The



primers used for the amplification of  $\alpha$ -SMA were: sense primer 5'-GGAG-CACCTGAGGACATTGAC-3' and antisense primer 5'-GCTTCAGTCAG-CAGAGTTGGG-3'. RT-PCR products were analyzed by electrophoresis in 1.5% agarose and the density of the bands was measured by densitometry using Glyko BandScan.

### Wounding experiment

Full-thickness excision wounds ( $\sim 0.5 \times 0.5$  cm) were made using a scalpel blade on the underside of the wings of 2-wk-old chicks. The left wing was treated with vehicle alone (water) and the right wing with  $1 \mu\text{g}$  cCAF (90 nM). The wounds were photographed immediately after wounding and then covered with Biocclusive bandage.  $50 \mu\text{l}$  vehicle (water) or cCAF was deposited through the bandage onto the wound using a 30-gauge needle. This procedure was repeated the next day and every other day thereafter. On days 3, 5, and 7 the bandages were removed and the wounds were photographed before replacing the bandages and applying the treatment again. Wings were placed on a flat surface at a distance of 25 cm from the digital camera and were always photographed with the same camera settings. All images were printed at the same magnification.

### Preparation and staining of wing sections

At the specified time points, chickens were killed with sodium pentobarbital. The wounded wings were collected and fixed for 18 h in 4% paraformaldehyde and decalcified for 3 d in 5% formic acid, 2.5% formaldehyde at 4°C. The tissue was embedded in paraffin and sectioned. Sections were stained with Masson Trichrome to visualize interstitial collagen. Other sections were immunolabeled for  $\alpha$ -SMA. Sections were deparaffinized three times with 15 min washes in Hemo-De and rehydrated in 5 min washes of ethanol (100, 95, 70, 50, and 30%). After rinsing with PBS, sections were fixed in 2% paraformaldehyde 1 h. Autofluorescence and nonspecific staining was blocked by 30 min in 0.1 M glycine in PBS, followed by 30 min in 1% Evans Blue in PBS to quench red blood cell autofluorescence. Sections were incubated with  $\alpha$ -SMA antibody in 1% BSA in PBS (1:50) for 2 h at room temperature. After three 10 min washes in 0.1% BSA in PBS, the sections were incubated for 40 min with anti-mouse Alexa antibody in 1% BSA in PBS (1:200). They were then washed and mounted with Vectashield. To quantify the number of myofibroblasts in the wounds, the number of fluorescently labeled cells in four high power fields of each wound was counted and statistical analysis was applied.

### Statistical methods

Significance was determined using Student's *t* test for comparison between two means and ANOVA for comparison between more than two means. All data were examined to assure homogeneity of variance. Means were considered significantly different when  $P < 0.05$ .

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